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RAPID COMMUNICATION

In situ hybridization and localization of mRNA for the rabbit prostaglandin EP_3 receptor

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***In situ* hybridization and localization of mRNA for the rabbit prostaglandin EP_3 receptor.** The physiological effects of PGE_2 appear to be mediated by at least three different "E-prostanoid" receptors designated EP_1, EP_2, and EP_3. These receptors are differentially activated by structural PGE analogs (such as misoprostol) and each couples to a different signal transduction mechanism. Studies demonstrating that inhibition of water absorption in the collecting duct is mediated by a G_i coupled mechanism, suggests that an EP_3 receptor is involved the renal effects of PGE_2. We used *in situ* hybridization to determine the tissue distribution of the rabbit EP_3 receptor. [α-^{35}S] UTP labeled antisense RNA, comprising transmembrane domains IV through VII, was hybridized to tissue sections. Specific labeling of kidney, stomach and adrenal was observed. In the kidney, medullary thick ascending limb and cortical and medullary collecting ducts were intensely labeled, while no labeling of glomeruli, proximal tubules, or cortical thick ascending limbs was observed. The adrenal gland labeled exclusively in the medulla. In the stomach the gastric epithelial crypts were the predominant site of hybridization, without evidence of labeling of the smooth muscle. These results suggest an important role for the EP_3 receptor in mediating PGE_2 effects in these tissues.

Prostaglandin E_2 (PGE_2) modulates an impressive array of physiologic responses. These effects include regulation of vascular tone, adrenal catecholamine release, gastric acid secretion, and modulation of renal salt and water transport. In the kidney, PGE_2 modulates glomerular hemodynamics [1–3], inhibits Na^+ transport in the thick ascending limb [4, 5], and inhibits both water and Na^+ transport in the collecting duct [6–9]. Under some circumstances its cellular effects appear to be self-opposing. For instance, when administered to the isolated perfused cortical collecting duct, PGE_2 stimulates water permeability and cyclic AMP generation [8, 10, 11], however, when administered to vasopressin pretreated collecting ducts PGE_2 inhibits water permeability and cyclic AMP generation [8, 10, 11]. Similarly PGE_2 has been shown to vasodilate certain vascular beds while it constricts other vascular beds [12–14]. These self-opposing effects of PGE_2 appear to be mediated by separate classes of PGE_2 receptors [15].

To date three receptor PGE_2 subtypes have been proposed, and designated as "E-prostanoid" receptors: EP_1, EP_2 and EP_3. Each is selectively activated by specific structural analogs of PGE_2 and is coupled to a different signal transduction mechanism. In the past year nucleotide sequences for the EP_3 and EP_2 receptor subtypes have been reported [16, 17]. The EP_3 receptor couples to an inhibitory G-protein (G_i) and inhibits cAMP generation while the EP_2 receptor couples to G_s and stimulates cAMP generation. The EP_1 receptor is thought to couple to phosphatidylinositol hydrolysis [18]. The existence of multiple PGE_2 receptors may account for its diverse effects.

The precise EP receptor subtype mediating the effects of PGE_2 in the kidney and other target tissues remains unclear. We have recently cloned and expressed the rabbit EP_3 receptor. Those studies demonstrated especially high levels of expression in the rabbit kidney, adrenal and stomach [19]. The purpose of the present studies was to localize this receptor subtype in these tissues.

Methods

Generation of antisense RNA fragments

For generation of antisense RNA probes, a fragment comprising nucleotides 565 to 1022 of the coding region of the rabbit EP_3 receptor [19] was amplified by PCR and ligated in the transcription vector pCRII. This region corresponds to amino acids comprising the fourth transmembrane domain to the seventh transmembrane domain. This region is common to all four of the cloned EP_3 subtypes [19]. The plasmid was linearized and antisense RNA was transcribed from the flanking T7 promoter in the presence [α-^{35}S] UTP essentially as described [20]. Antisense RNA (5×10^5 cpm/μl) was used for in situ hybridization.

Tissue preparation. Female New Zealand white rabbits weighing between 1.5 and 2 kg were anesthetized using intramuscular ketamine and xylazine (44 mg and 10 mg/kg, respectively). After surgical anesthesia was achieved, rabbits were sacrificed by decapitation and kidney, stomach, and adrenal were harvested. Tissues were fixed in 4% paraformaldehyde. The only exception to this were the kidneys which we initially perfused with a 1% paraformaldehyde-phosphate buffered saline prior to immersion in 4% paraformaldehyde. Tissues were imbedded in paraffin and 7 μm sections were cut.

Prior to hybridization sections were deparaffinized, re-fixed in paraformaldehyde and treated with proteinase K (20 μg/ml),

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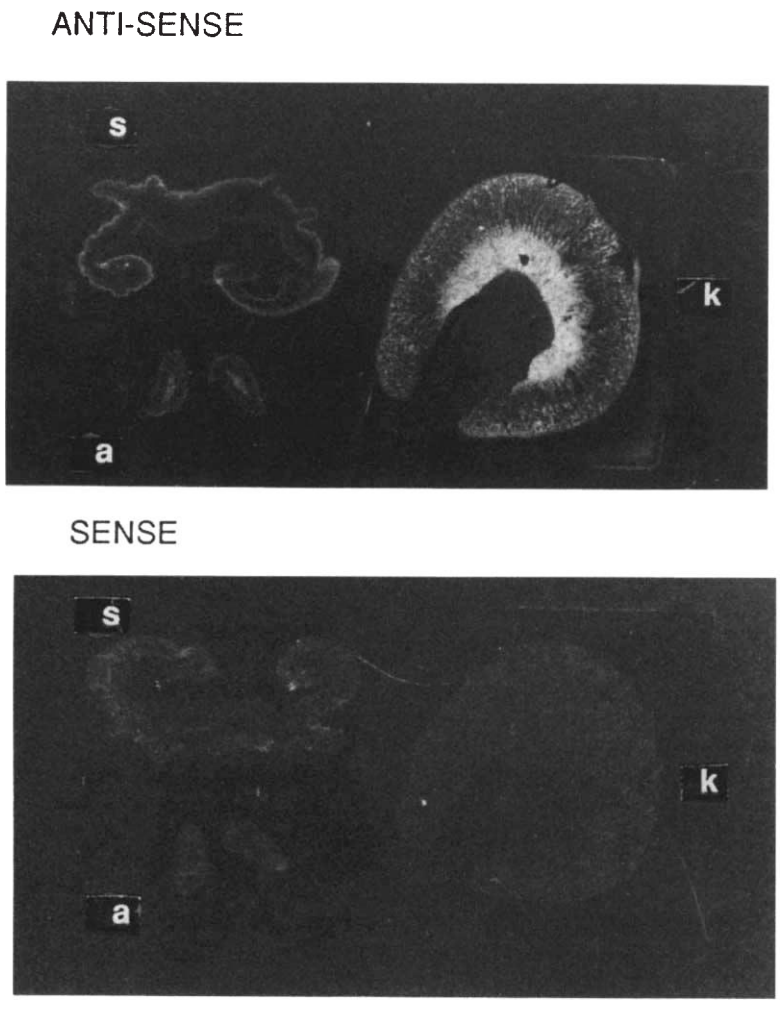


Fig. 1. *Autoradiograms of kidney (k), stomach (s), and adrenal (a) sections after hybridization with [α-^{35}S]UTP labeled EP_3 receptor anti-sense or sense RNA.*

washed with phosphate buffered saline, refixed in 4% paraformaldehyde, and treated with triethanolamine plus acetic anhydride (0.25% vol/vol). Finally sections were dehydrated to 100% ethanol.

Anti-sense RNA was hybridized to the sections at 50 to 55°C for approximately 18 hours as described by Pelton et al [21]. Following hybridization sections were washed at 50°C in 5× SSC + 10 mM β-mercaptoethanol for 30 minutes. This was followed by a wash in 50% formamide, 2× SSC, and 100 mM β-mercaptoethanol for 60 minutes. Following additional washes in 10 mM TRIS, 5mM EDTA, 500 mM NaCl (TEN), sections were treated with RNase (10 μg/ml), for 37°C, 30 minutes, followed by another wash in TEN (37°C). Sections were then washed twice in 2× SSC; and twice in 0.1× SSC (50°C). Slides were dehydrated with graded ethanols containing 300 mM ammonium acetate.

For detection of the hybridized probe, slides were dipped in photoemulsion (Ilford K5, Knutsford, UK) diluted 1:1 with 2% glycerol/water and exposed for seven days at 4°C. After development in Kodak D19, slides were counterstained with hematoxylin and eosin. Photomicrographs were taken using a Zeiss Axioskop using both bright and dark field optics.

Immunostaining

To define the nephron segments which labeled for EP_3 receptor RNA, in situ hybridization was followed by immunostaining of the tissue sections with an mouse monoclonal anti-collecting duct antibody [22] or a goat anti-human Tamm-Horsfall antibody, which specifically recognizes medullary and cortical thick ascending limb as well as the early portion of the distal tubule [23]. Tissue sections were incubated with serial dilutions of the anti-collecting duct or Tamm-Horsfall antibody (1:250, 1:1,000, 1:1,500, and 1:2,000). Immunolabeling was detected using either a biotinylated rabbit anti-goat or rabbit anti-mouse secondary antibody followed by visualization with an avidin-biotin horseradish peroxidase labeling kit (Vectastain ABC kit) and diaminobenzidine staining.

Autoradiography

In some cases the radiolabeled probe was detected by autoradiography rather than a photo-emulsion. For these studies the probe was allowed to hybridize to the tissue sections and following extensive washing the tissue was apposed to Kodak

Fig. 2. *Photomicrographs of in situ hybridization of kidney sections for EP_3 receptor mRNA.* A and B show 50× darkfield photomicrographs of rabbit kidney cortex and medulla respectively (the white and pink areas are the labeled structures). In **A**, the arrow is pointing along a line of unlabeled glomeruli. In figure **B**, OM is outer medulla and IM is inner medulla (papilla). **C** and **D** show 400× brightfield photomicrographs of cortex and medulla, respectively. The black grains indicate positive labeling for EP_3 receptor. The brown reaction indications positive staining with a mouse monoclonal anti-collecting duct antibody. Both cortical and medullary collecting ducts are labeled. **E** and **F** show 400× bright field photomicrographs of cortex and medulla respectively. The brown precipitate indicates positive staining with a goat anti-human Tamm-Horsfall antibody. In the cortex EP_3 mRNA was not detected in the thick limb (Tamm-Horsfall positive), while in the medulla both Tamm-Horsfall positive and Tamm-Horsfall negative tubules were labeled. **G** shows a 100× simultaneous brightfield/darkfield photomicrograph demonstrating different distribution of the EP_3 receptor mRNA (in situ grains appear white) and Tamm-Horsfall immunoreactivity (appears orange) in the renal cortex. **H** The arrows point to labeling of a tubule subpopulation in the cortex which is negative for both collecting duct markers (brown staining tubules) and, as shown in 2G, negative for thick limb markers. These tubules may represent distal convoluted tubule or connecting segment. Publication of this figure in color was made possible by an educational grant from Merck and Company, Human Health Division.

(XAR) film at 4°C for one week. The film was then developed in an automatic film processor.

Results

Autoradiography

Autoradiograms of kidney, stomach and adrenal showed specific labeling with anti-sense but not sense EP_3 receptor RNA fragments (Fig. 1). Intense labeling of kidney, stomach, and adrenal was observed. In the kidney the most intense labeling was detected over the outer medulla. There was punctate labeling of the cortex and no detectable labeling of the inner medulla. Similarly hybridization to the adrenal gland showed selective localization over the adrenal medulla with no detectable hybridization over the adrenal cortex. Autoradiograms of the stomach suggested labeling primarily over the gastric epithelium rather than the smooth muscle.

Photo-microscopy

Kidney. Intense labeling of most tubules in the renal outer medulla was observed. Figure 2 shows that labeling included both Tamm-Horsfall and anti-collecting duct antibody positive tubules, demonstrating both outer medullary collecting duct and medullary thick ascending limb express EP_3 mRNA. There was no evidence hybridization of the EP_3 antisense fragment to the proximal straight tubules in the outer medulla. No labeling of papillary or inner medullary structures was observed.

Hybridization of the EP_3 probe to the renal cortex was less intense than the renal outer medulla. Tamm-Horsfall positive tubules in the cortex were not labeled. Labeling in the cortex primarily involved the superficial cortical collecting duct as was confirmed by co-staining with a monoclonal antibody to collecting duct (Fig. 2 C and D). There also appeared to be a subpopulation of labeled cortical tubules which did not stain for either thick limb or collecting duct specific markers (Fig. 2H). This suggests labeling of either the distal convoluted tubules or connecting segment. No labeling of proximal tubules or glomeruli was detected.

Adrenal. The EP_3 probe showed intense labeling of adrenal chromaffin cells of the adrenal medullae. No specific labeling of cells in the adrenal cortex was seen (Fig. 3).

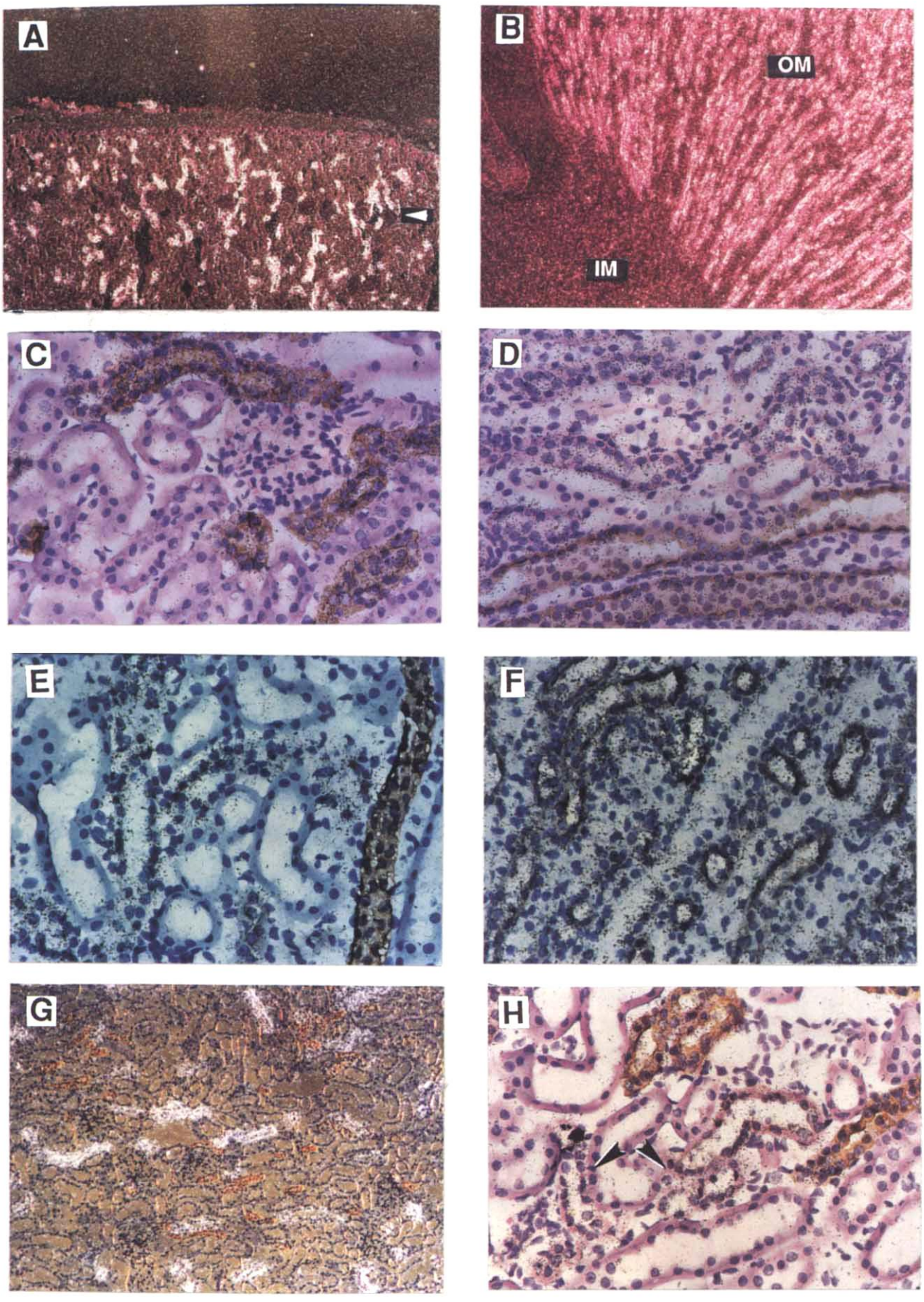
Stomach. Intense labeling of the gastric epithelium seen. This labeling was localized to the gastric crypts. The most superficial epithelium was unlabeled. No specific labeling of smooth muscle was observed (Fig. 3).

Discussion

It now appears that the disparate, and sometimes opposing effects of PGE_2, both at the signal-transduction and functional levels, are mediated by multiple receptors [14, 15]. These E-prostanoid (EP) receptors, have been classified as EP_1, EP_2 and EP_3. The cloned murine EP_3 receptor demonstrates discrete coupling to G_i, and inhibition of cAMP generation, without stimulation of inositol phosphate generation or cAMP accumulation [16]. More recently a putative mouse EP_2 receptor has been identified and shown to selectively stimulate cAMP generation [17]. The EP_1 receptor is thought to selectively stimulate phosphatidylinositol hydrolysis and increase intracellular calcium [18, 24].

Although it is well established that PGE_2 modulates function in the kidney [3, 10, 25], stomach [26, 27], and adrenal gland [28], the specific EP receptor isotype mediating these effects is unclear. Many of these functional effects of PGE_2 have been described in the rabbit [5, 11, 26, 27, 29, 30]. The present studies suggest the effects of PGE_2 on these rabbit tissues may involve an EP_3 receptor.

EP_3 receptor mRNA was expressed in the medullary thick ascending limb but was not detected in the cortical thick ascending limb. EP_3 mRNA was also expressed in both medullary and cortical collecting duct. No specific hybridization of EP_3-antisense RNA to glomeruli or proximal tubules was noted. The labeling of medullary but not cortical thick ascending limb is consistent with the studies of Stokes, who found that PGE_2 inhibits chloride absorption in the rabbit medullary thick ascending limb but had no effect on chloride transport in the cortical thick ascending limb of Henle [5]. Some studies have suggested an EP_3 receptor may be present in the rat cortical thick ascending limb [31, 32], however, significant differences in the hormonal regulation of nephron function exist between the rat and rabbit (including differing effects of PGE_2) [23, 33] so EP_3 receptor may be expressed in the cortical thick ascending limb of the rat but not the rabbit. Alternatively, and despite studies showing inhibition of transport in the medullary but not cortical thick ascending limb [5], other studies found no difference in the effects of PGE_2 on cAMP metabolism in the cortical and meduallary thick limb [34]. Of note, these latter studies, were limited to the juxtamedullary portion of the thick limb [34, 35], possibly accounting for this discrepancy, whereas the microdissection studies included the entire cortical thick limb. While we cannot exclude the presence of low levels of EP_3 mRNA in the cortical thick ascending limb, it seems clear that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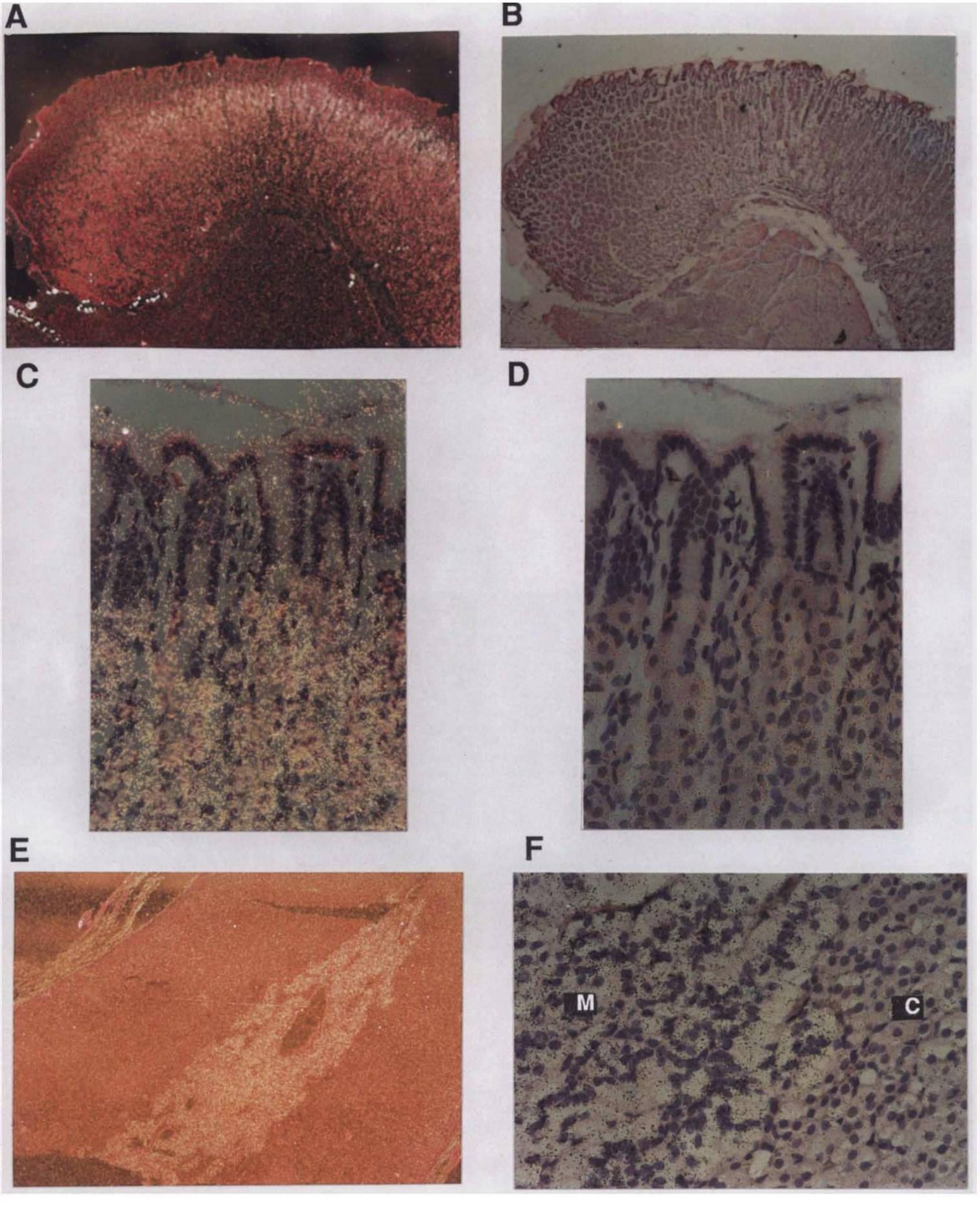
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Fig. 3. *Stomach and adrenal.* In the 50× darkfield photomicrograph of the stomach (**A**) the white grains indicate areas positive for EP_3 mRNA. **B** shows the same area of stomach under brightfield illumination. **C** and **D** show 400× simultaneous brightfield and darkfield illumination of gastric epithelium. In C the white grains demonstrates labeling of the crypts. In D darkfield illumination was decreased so the histologic features could be more clearly distinguished. **E** and **F** show the adrenal. E is a 50× darkfield photomicrograph where the white grains depicting positive labeling of the adrenal medulla. F is a 400× brightfield photomicrograph of the junction between adrenal medulla (M) and cortex (C). The black grains indicate the presence of EP_3 mRNA in the adrenal medulla. Publication of this figure in color was made possible by an educational grant from Merck and Company, Human Health Division.

the expression of this message is markedly less than in the mTAL, CCD or OMCD. The similarity between the functional effects of PGE_2 in microdissected rabbit cortical and medullary thick ascending limb, and the distribution of EP_3 mRNA along the thick ascending limb, suggests that the EP_3 receptor may play an important role in mediating the effects of PGE_2 on Cl^- transport in the mTAL.

Similarly the presence of EP_3 receptor mRNA in cortical and medullary collecting duct is consistent with previous studies demonstrating that the EP_1/EP_3 selective prostaglandin analog, sulprostone, potently inhibits vasopression stimulated water permeability and cAMP generation in the rabbit cortical collecting duct [11, 22, 29, 36]. Interestingly, there appeared to be a gradient for the intensity of EP_3 mRNA along the collecting duct, with greater levels of expression in the superficial, rather than the deep CCD. There was also substantial expression of EP_3 mRNA in the outer medullary collecting duct but little labeling of the papillary collecting duct. These observations are consistent with autoradiographic mapping of PGE_2 binding sites in human and rat kidney where specific [^{3}H]-PGE_2 binding was primarily observed in the outer medulla [37, 38] including the collecting duct and thick ascending limb. In agreement with the present studies, no [^{3}H]-PGE_2 binding to glomeruli or proximal tubule was seen. These [^{3}H]-PGE_2 binding studies did not address which EP receptor subtype(s) accounted for binding. The present studies suggest an EP_3 receptor may constitute the majority of these binding sites. In agreement with this possibility are biochemical studies suggesting that the major PGE_2 receptor in canine renal medulla is coupled to G_i [39]. Explicit examination of the distribution of the EP_3 receptor protein will have to await the development of EP_3 receptor-specific antibodies.

The present studies also suggest an important role for the EP_3 receptor in modulating gastric and adrenal function. PGE_2 stimulates catecholamine release in the adrenal medulla. This mechanism appears to involve both pertussis toxin insensitive phosphatidylinositol hydrolysis and pertussis toxin sensitive G_i coupled mechanisms [28]. The latter mechanism is consistent with a functional role for the EP_3 receptor in modulating catecholamine release from the adrenal medulla. Similarly, while specific PGE_2 binding sites have been identified in rabbit gastric glands [27] the EP receptor subtype which mediates its effects on acid secretion has not been characterized. Activity of PGE_2 analogs, such as misoprostol, in peptic ulcer disease depend on the nature of the EP receptor coupled to this functional effect. PGE_2 inhibits acid secretion by rabbit gastric glands via a pertussis toxin sensitive mechanism, coupled to the inhibition of cAMP generation [26]. Together with the present studies demonstrating the existence of EP_3 messenger RNA in gastric epithelial cells, these observations strongly suggest an important role for the EP_3 receptor in mediating PGE_2's effects on gastric acid secretion.

In summary the present studies demonstrate high levels of expression of the mRNA for the EP_3 receptor in renal and gastric epithelia. This distribution of EP_3 receptor mRNA corresponds to the nephron segments where PGE_2 has been demonstrated to inhibit NaCl and water transport. High levels of EP_3 receptor mRNA were also present in the adrenal medulla. Characterization of EP receptor subtype tissue distribution should help predict the physiologic consequences of their activation, and should help guide the therapeutic uses of receptor selective PGE_2 analogs, as they become increasingly available.

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